Dihydrotestosterone Inhibits Tumor Necrosis Factor α Induced Interleukin-1α mRNA Expression in Rheumatoid Fibroblast-Like Synovial Cells

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Rheumatoid arthritis (RA) is a chronic inflammatory disease that affects multiple synovial joints. Proinflammatory cytokines, such as interleukin-1 (IL-1) and tumor necrosis factor (TNF)α play important roles as principle inflammatory and destructive components of the disease. RA is known to be associated with significant gender differences in its prevalence and clinical features. We found that a potent androgen, 5α-dihydrotestosterone (DHT) inhibits IL-1α mRNA expression induced by TNFα and the DHT effect was inhibited by an androgen receptor antagonist, hydroxyflutamide (OHF). DHT inhibited the NF-κB activation induced by TNFα in a manner dependent on the androgen receptor (AR). These results suggest that DHT inhibits the TNFα-induced IL-1α mRNA expression by inhibiting NF-κB activation, and contributes to the gender differences of the disease.

Key words rheumatoid arthritis; synoviocytes; interleukin-1 (IL-1); tumor necrosis factor (TNF)α; androgen; androgen receptor

Rheumatoid arthritis (RA) is an autoimmune disease that is characterized by the chronic inflammation and hyperproliferation of synovial cells in multiple joints. Proinflammatory cytokines and chemokines produced by synoviocytes and infiltrated immune cells are implicated in the disease pathogenesis, such as production of proteases and reactive oxygen intermediates, proliferation of synovial fibroblasts, cartilage degradation, infiltration of inflammatory cells and angiogenesis.1 Antibody against TNFα, soluble receptor for TNFα, interleukin-1 receptor antagonist (IL-1ra) and antibody against IL-6 appeared to be effective in the treatment of RA patients, indicating that these cytokines are pivotal in the pathogenesis of the disease.2–5 These cytokines form a network, such as the induction of IL-1 and IL-6 by TNFα, and IL-6 induction by IL-1.6 Fibroblast-like synoviocytes are major cells that produce IL-1 and IL-6, and TNFα is probably produced by macrophages.7

There are two types of IL-1, IL-1α and IL-1β. They bind to the same receptor, type I IL-1 receptor, and transduce intracellular signaling.6 IL-1 contributes to the pathogenesis of the disease by affecting fibroblast-like synovial cells and cartilage to induce matrix metalloproteases (MMPs), NO and prostaglandin E2, which accelerate cartilage degradation. IL-1 also inhibits the synthesis of type II collagen from cartilage.8–10 By the experiments using animal model of arthritis IL-1α appeared to be important in the early phase of arthritis and participates in the inhibition of proteoglycan synthesis.11,12

Although it is not clarified how and what causes RA, RA is deviated to women as also observed in other autoimmune diseases; the ratio of RA incidence in women to men is about 4 : 1. The levels of estrogen to androgen in synovial fluid are elevated in both male and female RA patients13 and the level of testosterone in serum and synovial fluid is lower in male RA patients as compared to normal individuals.14 Therefore, sex hormones are implicated in the gender difference of RA.

In this study, we examined the effect of 5α-dihydrotestosterone (DHT) on the induction of IL-1α mRNA by TNFα, and suggested that DHA inhibits the effect of TNFα by inhibiting NF-κB activation in a manner dependent on the androgen receptor (AR).

MATERIALS AND METHODS

Reagents Dulbecco’s modified Eagle’s medium (DMEM) was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.); fetal bovine serum (FBS) was from JRH Bioscience (Lenexa, KS, U.S.A.); 5α-dihydrotestosterone (DHT) was from Fluka (Buchs, Switzerland). Hydroxyflutamide (OHF) was kindly provided by Nippon Kayaku Co., Ltd. (Tokyo, Japan). Human recombinant TNFα was provided by Dainippon Pharmaceutical Co. (Osaka, Japan). The specific activity of TNFα was 2.55×10 6 U/mg based on the cytotoxic assay using L929 cells cultured with actinomycin D.

Cell Culture Primary synovial cells from RA patients as well as MH7A, an immortalized cell line established by stably transfecting rheumatoid synoviocytes with the SV40 T antigen gene,15 were cultured in DMEM, with 100 U/ml of penicillin G, 100 μg/ml of streptomycin, 4 mM l-glutamine and 10% heat-inactivated FBS at 37°C in air containing 5% CO2. This study has been approved by the ethics committee of Kitasato University and Nagoya City University.

Plasmids pGL3-κBwt and NF-κB mutant construct

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pGL3-xB (for NF-κB reporter gene assay) have been described previously.\(^6\) pcDNA3-F-AR (AR expression vector) was constructed as described previously.\(^7\)

**Transient Transfection and Luciferase Assays** The day before transient transfection of MH7A cells, the culture medium was replaced by phenol red-free DMEM supplemented with 10% heat-inactivated FBS that had been pre-treated with dextran-coated charcoal to remove endogenous sex hormones. NF-κB reporter plasmid, AR expression plasmid and pCMV-βgal plasmid (for normalization of transfection efficiency) were transiently transfected into MH7A cells using the calcium phosphate-DNA co-precipitation method. After 16 h of transfection, cells were incubated with DHT for an additional 24 h and harvested. Luciferase assays were performed with the luciferase reporter gene assay kit (Roche, Germany) according to the manufacturer’s instructions. The light emission was measured using a multilabel counter 1420 ARVO (Perkin Elmer, Wellesley, MA, U.S.A.). Luciferase activity was expressed after normalization with the β-galactosidase value in the same sample.

**Semiquantitative Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) Analysis** Total RNA from cells was extracted according to the method of Chomczynski and Sacchi.\(^18\) The RT-PCR analysis was performed as described previously.\(^9\) Two micrograms of total RNA were reverse-transcribed with 50 mm Tris–HCl, pH 8.3, 75 mm KCl, 3 mm MgCl\(_2\), 10 mm DTT, 0.5 mm dNTP, 40 ng random primer, p(dN)\(_6\), 6 U ribonuclease inhibitor, and 40 U Maloney murine leukemia virus (MMLV) RT. One-tenth of the reversely-transcribed materials was used in the PCR reactions. Primers used for human IL-1α were 5’-ATGGAATGTCACATGATG-3’ and 5’-ATGGCTGTTTTCATATGTGTC-3’ (816-bp product); for human GAPDH were 5’-TGAAGGTGAGTTCAACCTGCT-3’ and 5’-CATGATGTCACCTGGCTCCAC-3’ (980-bp product). PCR were conducted as following conditions; 94 °C, 10 min; 30 cycles (human IL-1α or 24 cycles (human GAPDH) of 94 °C for 1 min, 54 or 56 °C for 1 min and 72 °C 1 min.

**RESULTS**

**DHT Inhibits the TNFα-Induced IL-1α mRNA Expression in Rheumatoid Synoviocytes** To investigate the effects of TNFα and DHT on the IL-1α mRNA expression in RA synovial cells, three primary preparations of fibroblast-like synovial cells from RA patients were treated with or without TNFα in the presence or absence of physiological concentration of DHT; serum level of total testosterone is 20.10 ± 4.50 and 19.10 ± 4.50 nmol/l in normal men and RA men, respectively. In women, serum testosterone is 0.78 ± 0.27 normal women and 0.20 ± 0.60 nmol/l in RA patients, respectively.\(^20\) RT-PCR analysis indicated that TNFα induced IL-1α mRNA expression in these primary synovial cells, and DHT significantly inhibited the IL-1α mRNA inducing activity of TNFα (Fig. 1).

**Androgen Receptor Antagonist Recovers the DHT Inhibition of TNFα-Induced IL-1α mRNA Expression** We also determined the effects of TNFα and DHT on the expression of IL-1α mRNA in fibroblast-like synovial cell line MH7A. Similarly to primary synovial cells TNFα at either 10 or 1 U/ml induced IL-1α mRNA expression in MH7A cells and DHT inhibited the effect of TNFα (Fig. 2). To determine whether the inhibitory effect of DHT was mediated by AR, MH7A cells were treated with TNFα with or without DHT in the presence or absence of androgen receptor antago-
nist, hydroxyflutamide (OHF). As shown in Fig. 2B, OHF significantly reversed the inhibitory effect of DHT.

**DHT Inhibits the TNFα-Induced NF-κB Activation in a Manner Dependent on AR** NF-κB is a key transcription factor for induction of IL-1α mRNA. To determine whether DHT inhibits the NF-κB activity by TNFα via the AR, MH7A cells were transfected with the NF-κB-dependent reporter plasmid with or without the expression vector of AR, and treated with TNFα in the presence or absence of DHT. As shown in Fig. 3, TNFα induced luciferase activity from the reporter plasmid containing four wild type NF-κB binding sites, but not the reporter plasmid containing four mutated inactive NF-κB binding sites. DHT alone did not affect the NF-κB activity and DHT did not inhibit the TNFα-induced NF-κB activation without AR overexpression. However, in the presence of over-expressed AR, DHT significantly inhibited the TNFα-induced NF-κB activation.

**DISCUSSION**

This is the first report indicating the inhibitory effect of DHT on IL-1α mRNA expression induced by TNFα in fibroblast-like synoviocytes derived from RA patients and fibroblast-like synoviocyte line MH7A. The inhibitory effect of DHT was recovered by androgen receptor antagonist. In addition, the reporter gene assay revealed that NF-κB activation by TNFα was inhibited by DHT in the presence of over-expressed AR. We confirmed AR expression in synovial cells and MH7A (data not shown). As the effect of DHT was not observed in the absence of over-expressed AR, the expression level of endogenous AR may not be sufficient to exhibit its function against transfected reporter plasmid. NF-κB is a critical transcription factor for activation of the IL-1α gene. These results suggest that the inhibitory effect of DHT is due to the inhibition of NF-κB activation by TNFα via AR, although we do not rule out the possibility that other transcription factors are also affected by DHT/AR.

NF-κB forms a dimer with Rel family proteins and is maintained in the cytoplasm as inactive complexes with inhibitory proteins, called IκB. Stimulation of TNFα or IL-1 leads to the sequential activation of the adapter protein myeloid differentiation factor 88 (MyD88), the IL-1 receptor associated kinases (IRAKs), TRAF2 or TRAF6, and eventually, the IκB kinase complex (IKKα, β, γ). The IKK complex phosphorylates the IκBs, targeting them for ubiquitination and degradation by the proteasome. Degradation of IκB liberates NF-κB/Rel dimers which translocate to the nucleus and augment the expression of NF-κB-responsive genes, including, IL-1α, IL-1β, IL-8, TNFα, MMPs and adhesion molecules.

AR is a member of the group of four closely related steroid receptors, the other members of which are glucocorticoid receptor, mineralocorticoid receptor, and progesterone receptor. This group comprises a subfamily of the larger and more diverse family of nuclear transcription factors. AR can be divided into four domains, an N-terminal domain that is involved in transcriptional regulation, a DNA-binding domain, a hinge region and a C-terminal ligand-binding domain. The ligand penetrates into cells and binds to AR in the cytoplasm. The ligand-activated AR forms a homodimer, translocates into nucleus, binds to target DNA, interacts with coactivators and induces transcription of the genes.

The mechanism by which the DHT/AR inhibits NF-κB activation is unclear. In COS-1 cells elevated expression of RelA (p65) repressed AR-mediated transactivation accompanied by the weak interaction between AR and RelA, and in prostate cancer LNCaP cells DHT suppressed NF-κB activity accompanied by a slight increase of IκB level. In a preliminary study we observed that GHA/AR inhibited the TNFα-induced degradation of IκB. However, the precise mechanism how DHT/AR inhibit the NF-κB activation by TNFα in synovial cells remains to be elucidated.

We have found that estrogen augments the mRNA expression of IL-1α by activating transcription of the gene via estrogen receptor α. By treatments with IL-1ra or antibodies against IL-1α or IL-1β, both IL-1α and IL-1β appeared to contribute to the arthritis caused by the immunization with type II collagen or by immune complex in the mouse. In IL-1α transgenic mouse membrane-bound IL-1α from synovocytes appears to be critical for the induction of arthritis. Interestingly, the severity of arthritis was correlated with membrane bound IL-1α rather than serum IL-1α or IL-1β. Therefore, our study suggests that DHT is a negative regulator for the induction or pathogenesis of RA by inhibiting TNFα-mediated induction of IL-1α and contributes to the gender differences of RA.

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**REFERENCES**

(2003).
